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Original Articles

Bortezomib enhances the therapeutic efficacy of dasatinib by promoting c-KIT internalization-induced apoptosis in gastrointestinal stromal tumor cells

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ABSTRACT

Dasatinib-based therapy is often used as a second-line therapeutic strategy for imatinib-resistance gastrointestinal stromal tumors (GISTs); however, acquired aberrant activation of dasatinib target proteins, such as c-KIT and PDGFRβ, attenuates the therapeutic efficiency of dasatinib. Combination therapy which inhibits the activation of dasatinib target proteins may enhance the cytotoxicity of dasatinib in GISTs.

Bortezomib, a proteasome inhibitor, significantly inhibited cell viability and promoted apoptosis of dasatinib-treated GIST-T1 cells, whereas GIST-T1 cells showed little dasatinib cytotoxicity when treated with dasatinib alone, as the upregulation of c-KIT caused by dasatinib itself interfered with the inhibition of c-KIT and PDGFRβ phosphorylation by dasatinib. Bortezomib induced internalization and degradation of c-KIT by binding c-KIT to Cbl, an E3 ubiquitin-protein ligase, and the subsequent release of Apaf-1, which was originally bound to the c-KIT-Hsp90β-Apaf-1 complex, induced primary apoptosis in GIST-T1 cells. Combined treatment with bortezomib plus dasatinib caused cell cycle arrest in the G1 phase through inactivation of PDGFRβ and promoted bortezomib-induced apoptosis in GIST-T1 cells. Our data suggest that combination therapy exerts better efficiency for eradicating GIST cells and may be a promising strategy for the future treatment of GISTs.

Introduction

Gastrointestinal stromal tumors (GISTs) are the largest subset of mesenchymal tumors of the digestive tract [1,2]. GISTs, derived from interstitial cell of Cajal (ICC) or gastrointestinal mesenchymal stem cells, are distinguished from other mesenchymal tumors occurring in the digestive tract, such as leiomyoma, leiomyosarcoma, and schwannoma, by their aberrant expression of c-KIT (CD117), a class III receptor tyrosine kinase (RTK) [3–5]. Over the past 10 years, based on our understanding of the role of RTKs in the potential oncogenesis of GISTs, the implementation of tyrosine kinase inhibitors (TKIs) has revolutionized the therapeutic strategy for GISTs [6,7]. Imatinib, the first-line TKI in treatment of chronic myelogenous leukemia (CML), has been approved and widely applied for GIST therapy [8].

c-KIT (mammalian cellular homolog of *v-KIT* in Hardy–Zuckerman-feline sarcoma virus) proto-oncogene is a 145-kD transmembrane RTK, consisting of a extracellular ligand-binding domain, a single transmembrane domain, an intracellular juxtamembrane region, and a split intracellular kinase domain [9]. When the c-KIT ligand is bound, the stem cell factor (SCF) and two neighboring c-KIT proteins undergo dimerization followed by phosphorylation of tyrosine residues in the juxtamembrane region and kinase domains, which formatted as the dock sits of Src homology 2 (SH2) domains of a variety of signal molecules, including PI3K, AKT, ERK, and so on [9,10]. The activation of factors downstream of the c-KIT signaling cascade is extremely important for pro-oncogenic cellular progress, and specifically for anti-apoptosis

Abbreviations: Apaf-1, apoptotic protease activating factor 1; BOR, bortezomib; DAPI, 4′,6-diamidino-2-phenylindole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GISTs, gastrointestinal stromal tumors; Hsp90β, heat shock protein 90 β; PDGFRβ, platelet-derived growth factor receptor β; PI, propidium iodide; RTK, receptor tyrosine kinase; SCF, stem cell factor; TKIs, tyrosine kinase inhibitors.

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[11–13]. However, the precise underlying role of c-KIT involvement in the apoptosis cascade is rarely investigated.

Most cases of GISTs harbor a c-KIT mutation, most frequently in exon 11, which encodes a juxtamembrane region normally functioning in inhibiting c-KIT auto-phosphorylation [14,15]. Although c-KIT is the marker and target of GIST therapy, the remarkable heterogeneity due to mutations in different sites inhibits the general application of TKIs [16,17]. Among the different sites for c-KIT mutations, exon 11 is the most commonly influenced region and is associated with the best response to imatinib, whereas other mutation types indicate pre-clinical resistance to imatinib therapy [16–18]. In addition, half of the patients enjoying the initial benefits of imatinib therapy will develop resistance to imatinib within approximately 2 years, which is classified as delayed resistance [16]. Hence, a novel therapeutic strategy for GISTs is still needed for clinical practice.

Dasatinib (BMS-354825), a dual SRC/ABL inhibitor, is a multi-kinase inhibitor targeting the SRC family (SRC, LCK, YES, FYN), c-KIT, and platelet-derived growth factor receptor β (PDGFRβ) [19–21]. It has been approval for treating primary or secondary imatinib-resistance GISTs and wild-type GISTs (no mutations in either *c-KIT* or *PDGFRβ*). In addition, dasatinib shows promise as a TKI therapy for GISTs according to its higher binding capacity to c-KIT or PDGFRβ regardless of the conformation of the c-KIT activation loop [22,23]. However, like imatinib, the heterogeneity of c-KIT also imposes restrictions on the use of dasatinib as a GIST therapy [16,24]. For instance, only GISTs harboring certain mutations in the activation loop of c-KIT may benefit from dasatinib treatment [25,26]. Moreover, in CML and Philadelphia-positive (Ph+) leukemia, resistance to dasatinib has been proven to be associated with aberrant BCR/ABL expression or certain mutation types [27]. In this study, we found that upregulation of c-KIT induced by dasatinib significantly reduced the cytotoxicity of dasatinib in GIST-T1 cells. Therefore, pre-clinical research about how to overcome the obstacle of c-KIT heterogeneity for the clinical application of TKI is needed.

In order to prolong disease free survival in GIST patients, alternative TKIs or combined TKI therapeutic strategies with other chemotherapeutic agents targeting c-KIT or PDGFR or downstream factors of RTKs have shown potential for GIST treatment [28,29]. Hence, induction of degradation of c-KIT by chemo-agents may provide a new insight for treatment of GISTs. The cellular protein degradation systems including ubiquitin–proteasome system and lysosome system are intimately related to the apoptosis pathway due to their destruction of endogenous or exogenous anti-apoptosis proteins [30,31]. Previous research showed membrane receptor proteins are usually degraded trough ubiquitin and an E3 ubiquitin–protein ligase, Cbl-mediated lysosome pathway [32,33]. However, the precise mechanism by which c-KIT is degraded and how it regulates apoptosis in GISTs is still unknown. Recently, our previous research revealed that bortezomib (BOR), a proteasome inhibitor, was reported to regulate the c-KIT-involved apoptosis cascade in leukemia cells through inducing c-KIT internalization and lysosome-induced degradation [34]. Hence, in this study, based on evidence that upregulation of c-KIT induced by dasatinib influenced the therapeutic efficiency of dasatinib in GISTs, we aimed to investigate if the combination of dasatinib and BOR exerted a synergetic cytotoxicity on GIST cells.

Materials and methods

Cell culture

GIST-T1 cells were obtained from the Shanghai Institute for Biological Science (Shanghai, China) and cultured in RPMI 1640 (Gibco; Carlsbad, CA, USA) containing 10% (vol/vol) fetal bovine serum (FBS; Gibco), 100 U/mL penicillin, and 100 mg/mL streptomycin in a 5% (vol/vol) CO_2 atmosphere at 37 °C. Cells in the exponential growth phase were coincubated with dasatinib, BOR, and/or dynasore (DY) at indicated concentrations.

Drugs and antibodies

Dasatinib and BOR were purchased from Selleckchem (Houston, TX, USA) and DY was purchased from Sigma-Aldrich (St. Louis, MO, USA). Total-c-KIT, phospho-c-KIT (p-c-KIT), total-PDGFRβ, phospho-PDGFRβ (p-PDGFRβ), Cbl, total-heat shock protein (Hsp) 90β, and apoptotic protease activating factor 1 (Apaf-1) primary antibodies for western blot, immunoprecipitation, or immunofluorescence were obtained from Cell Signaling (Danvers, MA, USA). Phosphor-Hsp90β (p-Hsp90β; pTyr) antibody was purchased from Millipore (Billerica, MA, USA). Anti-His and anti-Flag primary antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). The GAPDH primary antibody and HRP-conjugated secondary antibodies were purchased from Kangchen Biotechnology (Shanghai, China). 4′,6-diamidino-2-phenylindole (DAPI; Sigma) and Alexa Fluor 488 (AF488) secondary antibody were both purchased from Invitrogen (Carlsbad, CA, USA).

Plasmids/siRNA transfections

Before transfection, c-KIT and Hsp90β were cloned into pcDNA3.1(−)-Flag vectors. Similarly, His-Apaf-1 was subcloned into a pcDNA3.1(−) vector from pFastBac-His-Apaf-1. GIST-T1 cells were transfected with plasmids or small interfering (si) RNAs using Lipofectamine-2000 (Invitrogen) for 6 hours and all experiments were performed in 72 hours after transfection according to manufacturer's instructions.

Western blot and immunoprecipitation analysis

GIST-T1 cell lysates were resuspended in cell lysis buffer (Cell Signaling). The protein concentrations were quantified using the BCA Protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL, USA). Prepared protein lysates were mixed with loading buffer, denatured by boiling, separated using 10% SDS–PAGE gels, and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). After blocking with Tris-buffered saline (TBS) and 0.1% Tween 20 (TBS/T) containing 5% bovine serum albumin for 2 hours, membranes were incubated with primary antibodies at 4 °C overnight. The membranes were incubated with HRP-conjugated secondary antibody for 2 hours at room temperature. Protein bands were developed by chemiluminescence (GE Healthcare; Piscataway, NJ, USA) and visualized using an autoradiography kit (Kodak; Rochester, NY, USA). For immunoprecipitation analysis, cell lysates pre-incubated with conditional antibodies were precipitated for 2 hours by pre-washed Protein A-Sepharose beads (Santa Cruz Biotechnology). The precipitated complex was washed, separated using 10% SDS–PAGE gels, transferred to PVDF membranes, and then incubated with anti-Cbl, anti-His, anti-Flag, anti-Hsp90β, anti-p-Hsp90β, anti-c-KIT, or anti-Apaf-1 primary antibodies and a HRP-conjugated secondary antibody.

Assessment of cell viability, proliferation and apoptosis

Cells were plated into 96-well plates cultured in media containing different drugs for indicated times. The cell viabilities were tested using the cell count kit-8 (CCK-8; Dojindo; Kumamoto, Japan) following the manufacturer's protocol. After incubation with CKK-8 solutions for 3 hours, the absorbance of each wells was then measured at 450 nm using a MRX II microplate reader (Dynex, Chantilly, VA, USA). Relative cell viability was determined as a percentage of untreated control cells. Calculation of the half maximal inhibitory concentration (IC_{50}) of different drugs were performed using Graphpad Prism (GraphPad Software, Inc.; La Jolla, CA, USA) and the combination index (CI) value of the combination of the two drugs was calculated using formula "$CI = (IC_{50})_{C\text{-}D}/(IC_{50})_D + (IC_{50})_{C\text{-}B}/(IC_{50})_B$", where $CI \le 0.9$, > 0.9 and <1.1, ≥ 1.1 indicate synergism, additive effect, and antagonism, respectively [35]. In the denominator, $(IC_{50})_D$ or $(IC_{50})_B$ is for the concentration of "dasatinib or BOR alone" that inhibits the cell viability by 50%. In the numerators, $(IC_{50})_{C\text{-}D}$ or $(IC_{50})_{C\text{-}B}$ means the respective IC_{50} of dasatinib or BOR "in combination" treatment. The proliferation of treated GIST-T1 cells was detected using Click-iT 5-ethynyl-20-deoxyuridine (EdU) Imaging Kit (Invitrogen) as described previously [36]. In brief, treated GIST-T1 cells were incubated with EdU for 2 hours followed by fixed with 4% paraformaldehyde for 15 minutes and permeabilized with 0.5% Triton X-100 for 20 minutes. Then cells were incubated with 1X Click-iT reaction cocktails for 30 minutes in dark. After stained by Hoechst 33342, cells were visualized using an inverted fluorescence microscope (Olympus, Tokyo, Japan). For quantification of treated GIST-T1 cell proliferation, five randomly selected views from each treatment were used to calculate the relative EdU-positive cell ratio. Cell apoptosis was evaluated using an Annexin V-FITC/PI kit (Invitrogen) for flow cytometry according to manufacturer's instruction.

Cell cycle analysis

Cell cycle analysis was performed by flow cytometry by staining GIST-T1 cell DNA with propidium iodide (PI; Dawen; Shanghai, China). Distribution of cell cycle was analyzed using the ModFit software (Verity software house; Topsham, ME, USA). In brief, treated cells were fixed by pre-chilled ethanol overnight and centrifuged before being incubation with PI.

Immunofluorescence microscopy

In brief, cells planted on slides were fixed with 4% paraformaldehyde for 15 minutes before being permeabilized in 0.5% Triton X-100 in phosphate buffered saline (PBS). Subsequently, cells were incubated in c-KIT primary antibody overnight and AF-488- conjugated secondary antibody for 2 hours followed by incubation with DAPI for 2 minutes. Immunofluorescence images were taken and captured using an inverted fluorescence microscope.

Statistical analyses

All experimental data are presented as mean and standard deviation (SD) values. Statistical analysis was performed using Prism5 (GraphPad). Student's *t*-test and the two-way ANOVA followed by Bonferroni's post hoc test were used to assess the significance of different treatments; statistical significance was defined as a *P*-value <0.05. Each treatment was assayed in triplicate.

Results

Upregulation of c-KIT induced by dasatinib treatment was responsible for dasatinib chemoresistance in GIST-T1 cells

We first assessed the response of GIST-T1 to dasatinib and phosphorylation patterns of c-KIT and PDGFRβ, the two main target TRKs of dasatinib, in the presence or absence of dasatinib in GIST-T1 cells. We found that p-c-KIT and p-PDGFRβ were highly expressed, which means that c-KIT and PDGFRβ were activated in GIST-T1 cells (Fig. 1A); however, as a multi-kinase inhibitor, dasatinib did not reduce the phosphorylation of c-KIT or PDGFRβ in GIST-T1 cells (Fig. 1A), and thus dasatinib did not significantly inhibit the cell viability of GIST-T1 cells (Fig. 1B). Interestingly, after 24 hours of treatment with dasatinib, the expression of c-KIT, but not PDGFRβ, was significantly up-regulated, which indicates that upregulation of c-KIT might result in chemoresistance to dasatinib in GIST-T1 cells. To confirm this, we artificially down-regulated the expression of c-KIT using *c-KIT* siRNA and found that dasatinib dramatically inhibited cell growth in *c-KIT* siRNA-treated GIST-T1 cells, the IC_{50} of dasatinib significantly downregulated in *c-KIT* siRNA-treated GIST-T1 cells (Fig. 1B; WT vs. *c-KIT* siRNA, $p < 0.001$, two-way ANOVA). Moreover, dasatinib also reduced the phosphorylation of PDGFRβ in *c-KIT* siRNA-treated GIST-T1 cells (Fig. 1A). This evidence indicates that upregulation of c-KIT induced by dasatinib might be responsible for chemo-resistance to dasatinib in GIST-T1 cells.

In addition, we found that the percentage of apoptotic ratio significantly increased in *c-KIT* siRNA GIST-T1 cells (Fig. S1A). And dasatinib significantly promoted the apoptosis induced by *c-KIT* siRNA; however dasatinib did not induce apoptosis in WT GIST-T1 (Fig. 1C; Fig. S1A). Cell cycle analysis showed that compared to wild type (WT) GIST-T1 cells, *c-KIT* siRNA GIST-T1 cells were arrested in G1 phase after treatment with dasatinib (Fig. 1C; Fig. S1B).

Combination treatment with bortezomib reversed dasatinib-induced upregulation of c-KIT and exerted synergetic cytotoxicity in GIST-T1 cells

To investigate if combination treatment could increase the sensitivity of GIST-T1 to dasatinib, we tested the viability of GIST-T1 cells treated with dasatinib alone, BOR alone and dasatinib plus BOR and then calculated the IC_{50} of dasatinib and BOR and the CI value of the combination of the two drugs (Table 1; Fig. 2A). Compared to dasatinib alone, combination treatment significantly inhibited cell viability (Fig. 2A). In addition, BOR reduced c-KIT and p-c-KIT expression, and the expression of c-KIT, p-c-KIT, and p-PDGFRβ were also significantly reduced after treatment with dasatinib plus BOR; however, BOR alone had little effect on the expression of p-PDGFRβ (Fig. 2B). The total expression of PDGFRβ did not significantly change following these treatments (Fig. 2B).

Furthermore, combined treatment did not exert a synergetic effect in *c-KIT* siRNA-treated GIST-T1 cells compared to dasatinib treatment alone (Fig. 2C; Table 1). In addition, when the expression of PDGFRβ was inhibited by *PDGFRβ* siRNA in GIST-T1 cells, combined treatment did not exert a synergetic effect compared to BOR alone (Fig. 2D; Table 1). An Edu incorporation assay was used to assess proliferation inhibition of GIST-T1 or *c-KIT*/*PDGFRβ* siRNA GIST-T1 cells treated with dasatinib, BOR, or dasatinib plus BOR. In agreement with CCK8 results, combination treatment significantly inhibited the proliferation of GIST-T1 cells compared to dasatinib alone (Fig. 2E; Fig. S2A); however, *c-KIT* siRNA or *PDGFRβ* siRNA significantly attenuated the synergetic effect of dasatinib plus BOR compared to dasatinib or BOR treatment, respectively (Fig. 2F; Fig. S2B).

c-KIT was endocytosed in a clathrin-mediated way and degraded by binding to Cbl after bortezomib treatment

To investigate how BOR resulted in down-regulation of c-KIT in GIST-T1 cells, we measured the expression of c-KIT after BOR treatment alone by western blot and immunofluorescence assay. We found that after BOR treatment for 24 hours, c-KIT and p-c-KIT expression were dramatically down-regulated (Fig. 3A) and the immunofluorescence analyses of c-KIT revealed that it was endocytosed and degraded at the late stage (24 hours) in GIST-T1 cells (Fig. 3B). Previous studies showed that internalization of c-KIT was mediated by clathrin; hence, we evaluated the role of clathrin in BOR-caused internalization of c-KIT using DY, an inhibitor of dynamin, which is essential for the formation of coated vesicles of clathrin-mediated internalization [37]. We found that incubation with DY inhibited the internalization and degradation of c-KIT induced by BOR (Fig. 3C) and retained c-KIT on the cell membrane in the presence of BOR in GIST-T1 cells (Fig. 3D). Moreover, DY reversed bortezomib-induced inhibition of cell viability and apoptosis (Fig. S3).

When c-KIT is endocytosed by BOR, it might undergo ubiquitination and degradation by binding to the ubiquitin ligase E3, among which the Cbl family is the most important. To verify the hypothesis that c-KIT was degraded by binding to Cbl, we performed a co-immunoprecipitation assay to test the interaction of Cbl and c-KIT in GIST-T1 cells. We found that without BOR treatment, c-KIT did not bind to Cbl; after 12 hours of BOR treatment, the expression of Cbl and the binding of c-KIT and Cbl was drastically up-regulated, which indicates that c-KIT had been modified by ubiquitin molecules and as a target protein followed by degradation (Fig. 3E). To confirm the role of Cbl in the degradation of c-KIT, *Cbl* siRNA was exerted and we found that BOR-induced downregulation of c-KIT was reversed by knockout of the *Cbl* gene in GIST-T1 cells (Fig. 3F; Fig. S4). This evidence shows that BOR down-regulates the expression of c-KIT through rendering the internalization of c-KIT, which was subsequently degraded by binding to Cbl in GIST-T1 cells.

Table 1
IC_{50} values and CI (combination index) for dasatinib and bortezomib in WT or *c-KIT*/*PDGFRβ* siRNA GIST-T1 cells.

Cell line	IC_{50} of Das (nM)[a]		IC_{50} of BOR (nM)[b]		Combination index
GIST-T1	Das	Das + BOR	BOR	BOR + Das	
WT	965.7	27.0***	130.0	26.9***	0.24
c-KIT siRNA	35.6	25.6**	104.7	25.4***	0.96
PDGFRβ siRNA	276.4	65.9***	76.3	66.2*	1.1

[a] IC_{50} of dasatinib (Das) in different treatments.
[b] IC_{50} of bortezomib (BOR) in different treatments.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

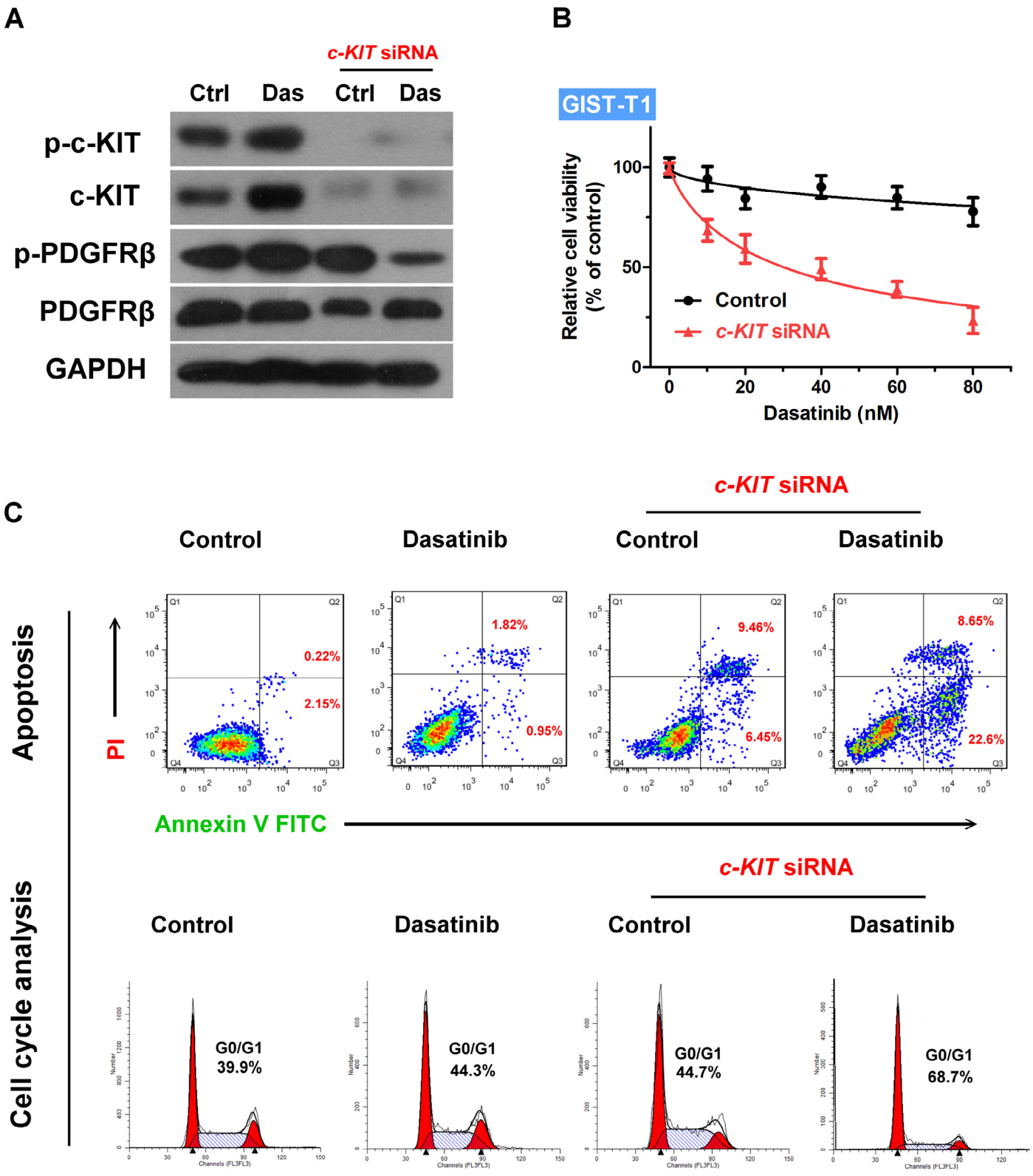

Fig. 1. Dasatinib exerted little effect on GIST-T1 cells as c-KIT expression was induced by dasatinib. (A) Western blot analyses of expression of phosphorylation and total c-KIT and PDGFRβ in GIST-T1 cells (Ctrl) and *c-KIT* siRNA GIST-T1 cells treated with or without dasatinib (Das). (B) Mean ± SD relative viability of GIST-T1 cells and *c-KIT* siRNA GIST-T1 cells treated with dasatinib in different concentrations. (C) Apoptotic and cell cycle analyses of GIST-T1 cells and *c-KIT* siRNA GIST-T1 cells treated with or without dasatinib.

c-KIT indirectly binds to Apaf-1 through Hsp90β

With respect to how c-KIT degradation contributes to the inhibition of proliferation of GIST-T1 cells, we speculated that c-KIT might interplay with pro-apoptotic factors and once c-KIT was internalized and degraded, these factors were released from the complex and propagated their downstream pathway. On the basis of our hypothesis and previous research [34], we tested the

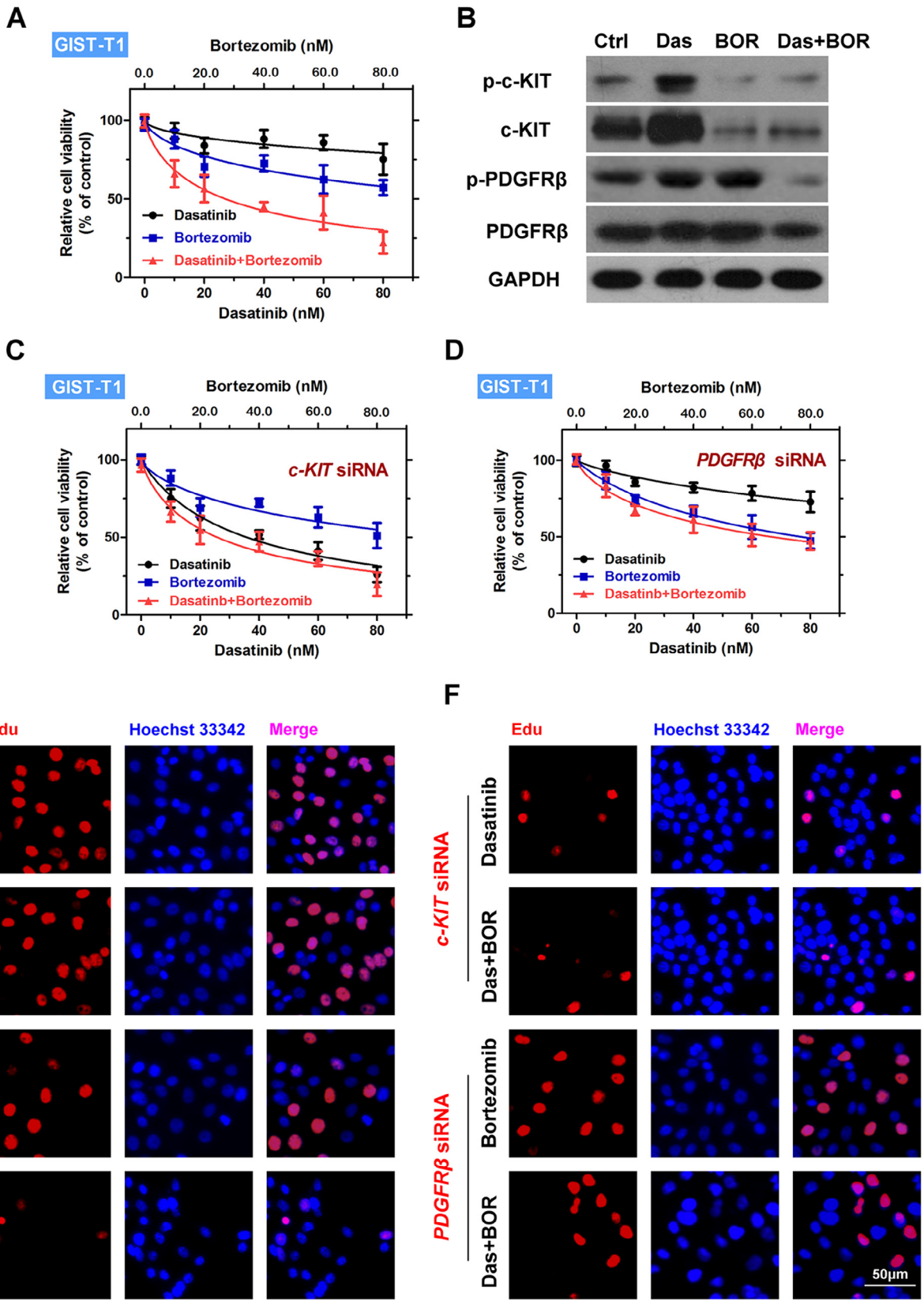

Fig. 2. Bortezomib enhanced the cytotoxicity of dasatinib in GIST-T1 cells. (A) Mean ± SD relative viability of GIST-T1 cells treated with dasatinib, bortezomib and dasatinib plus bortezomib. (B) Western blot analyses of phosphorylation and total of c-KIT and PDGFRβ expression in untreated GIST-T1 cells (Ctrl) and/or GIST-T1 cells treated with dasatinib (Das), bortezomib (BOR) and dasatinib plus bortezomib (Das + BOR). (C) Mean ± SD relative viability of *c-KIT* siRNA GIST-T1 cells treated with dasatinib, bortezomib and dasatinib plus bortezomib. (D) Mean ± SD relative viability of *PDGFRβ* siRNA GIST-T1 cells treated with dasatinib, bortezomib and dasatinib plus bortezomib. (E) Edu incorporation analyses of untreated GIST-T1 cells (control) and/or GIST-T1 cells treated with dasatinib, bortezomib, and dasatinib plus bortezomib. (F) Edu incorporation analyses of *c-KIT* siRNA GIST-T1 cells treated with dasatinib and dasatinib plus bortezomib (Das + BOR) and/or *PDGFRβ* siRNA GIST-T1 cells treated with bortezomib and dasatinib plus bortezomib (Das + BOR).

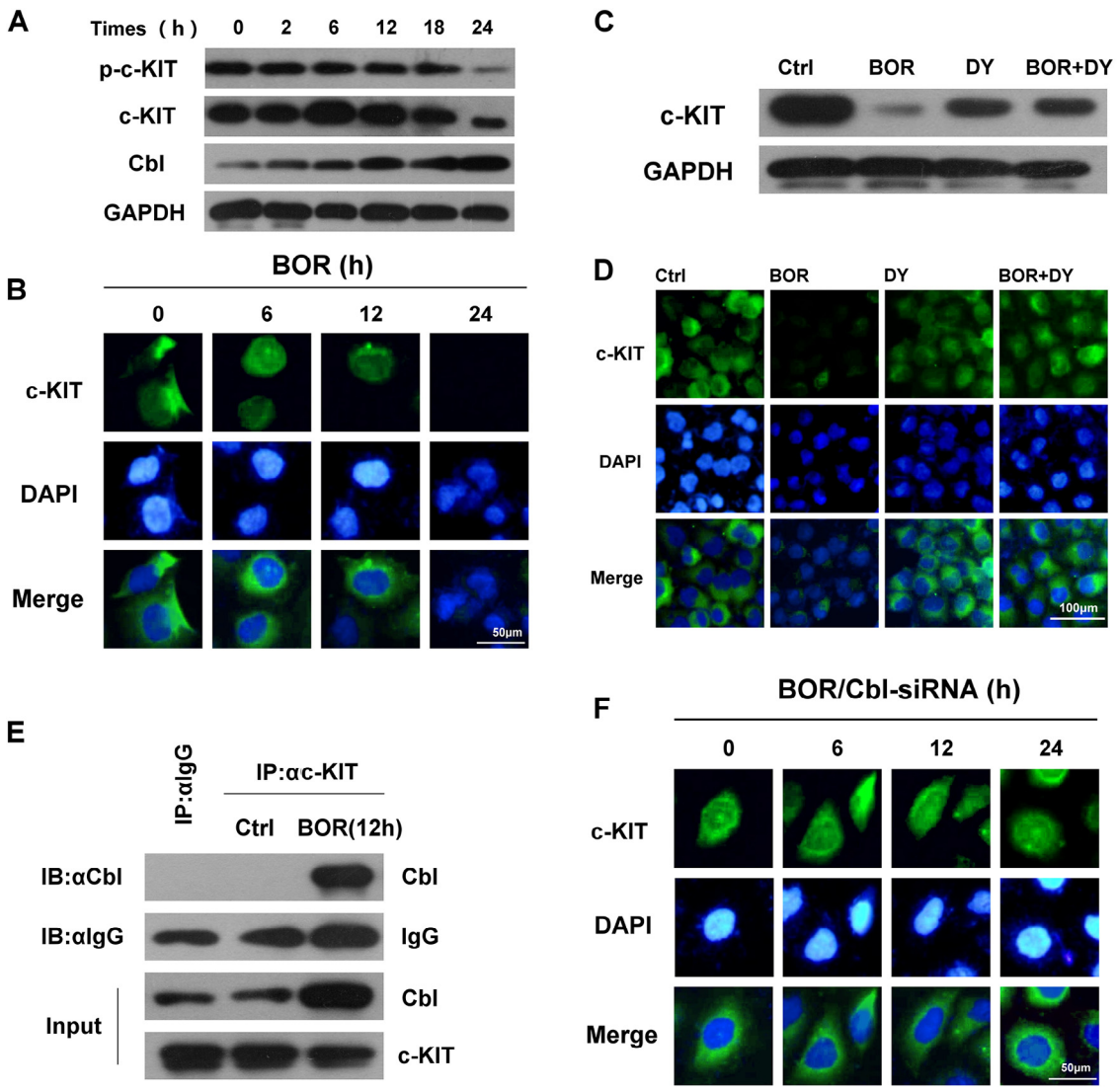

Fig. 3. Bortezomib triggered c-KIT internalization and subsequent degradation by binding to Cbl in GIST-T1 cells. (A) Western blot analyses of phosphorylation and total of c-KIT and Cbl expression in GIST-T1 cells treated with bortezomib for the indicated time points. (B) Immunofluorescence analyses of subcellular location and expression of c-KIT in GIST-T1 cells treated with bortezomib for the indicated time points. (C) Western blot analyses of c-KIT expression in untreated GIST-T1 cells (Ctrl) and/or GIST-T1 cells treated with bortezomib (BOR), DY, and DY plus bortezomib (DY + BOR). (D) Immunofluorescence analyses of subcellular location and expression of c-KIT in untreated GIST-T1 cells (Ctrl) and/or GIST-T1 cells treated with bortezomib (BOR), DY, and DY plus bortezomib (DY + BOR). (E) Co-immunoprecipitation analyses of c-KIT and Cbl binding in untreated GIST-T1 cells and/or GIST-T1 cells treated with bortezomib. (F) Immunofluorescence analyses of subcellular location and expression of c-KIT in *Cbl* siRNA GIST-T1 cells treated with bortezomib (BOR) for the indicated time points.

interaction of c-KIT and Hsp90β by co-immunoprecipitation assay in GIST-T1 cells. We transfected Flag-Hsp90β and/or Flag-c-KIT plasmids into 293T cells and found that c-KIT not only bound to Hsp90β, but also induced phosphorylation of Hsp90β, which represents its activated status (Fig. 4A). In GIST-T1 cells, BOR induced down-regulation of c-KIT and resulted in down-regulation of Hsp90β phosphorylation (Fig. 4B).

It has been reported that Apaf-1 has a high binding affinity for Hsp90β [38]. To confirm if Apaf-1 was the main factor in mediating BOR-induced inhibition of cell growth, the purified protein of 293T cells transfected with Flag-Hsp90β and His- Apaf-1 was incubated with c-KIT protein isolated from GIST-T1 cells and we performed reciprocal co-immunoprecipitation and western blot assays to investigate the relationship between c-KIT, Hsp90β, and Apaf-1. We found that c-KIT significantly enhanced the binding affinity of Hsp90β and Apaf-1 through inducing phosphorylation of Hsp90β (Fig. 4C). In GIST-T1 cells, BOR decreased phosphorylation of Hsp90β and released Apaf-1 from the complex (Fig. 4B). This evidence indicates that phosphorylation of Hsp90β induced by c-KIT is essential for Apaf-1 sequestration.

Dasatinib enhanced the apoptosis induced by bortezomib by cell cycle arrest in G1 in GIST-T1 cells

As Apaf-1 plays a role in promoting apoptosis [39], we determined GIST-T1 apoptosis status after treatment with dasatinib, BOR, and dasatinib plus BOR. We found that dasatinib alone could not trigger obvious apoptosis, and BOR only induced an approximately 10% apoptotic cell ratio in treated cells; however, the combination of dasatinib and BOR resulted in significant increase of percentage of apoptotic cell ratio (Fig. 5A; Fig. S1C). We also found that *PDGFRβ* siRNA relieved cell cycle arrest induced by dasatinib and attenuated dasatinib-induced promotion of apoptosis triggered by BOR in combination treatment (Fig. 5B; Fig. S1D and E).

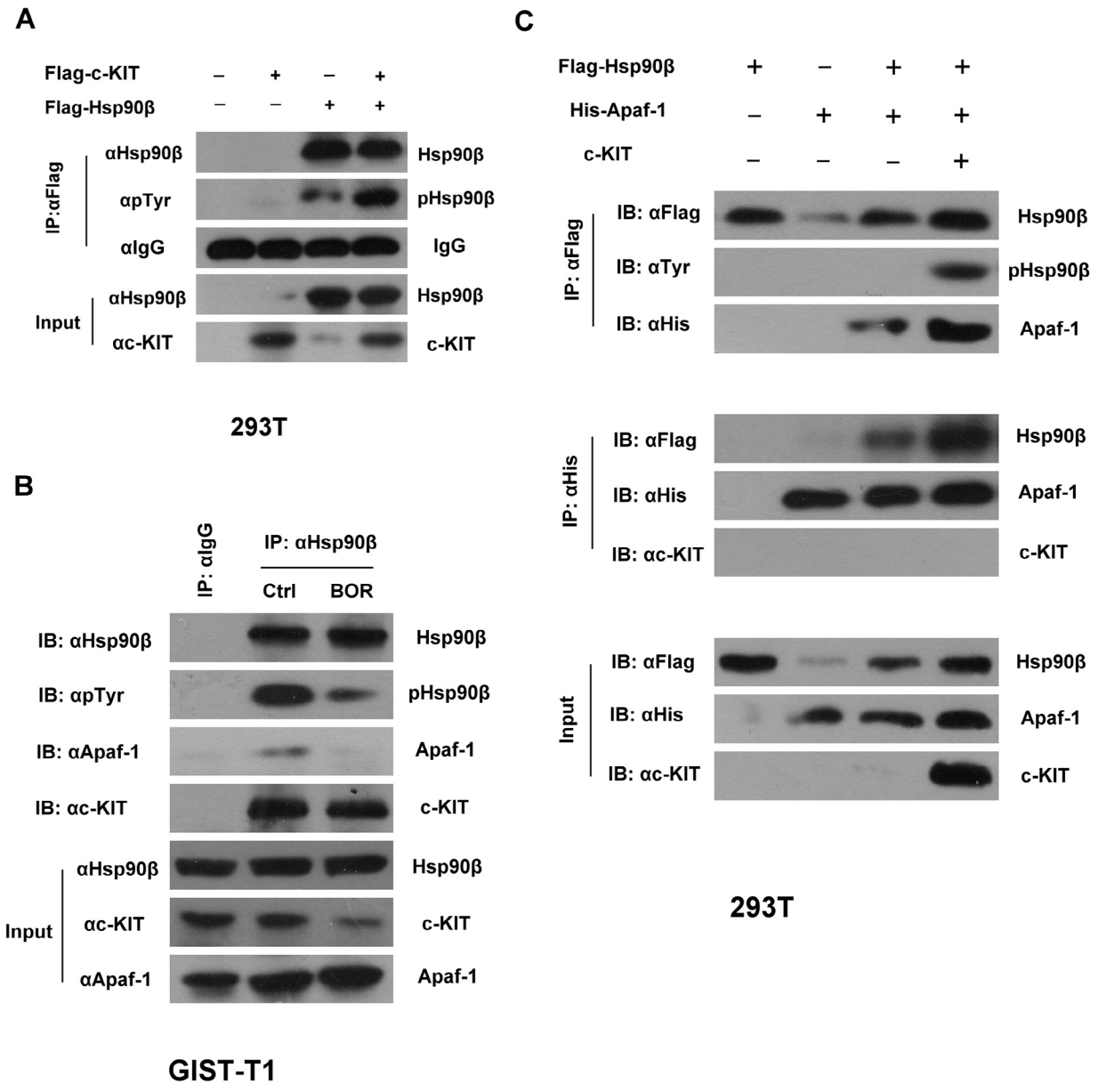

Fig. 4. c-KIT enhanced the binding affinity of Hsp90β and Apaf-1 through inducing phosphorylation of Hsp90β. (A) Co-immunoprecipitation analyses of c-KIT and Hsp90β/phosphor-Hsp90β (pTyr) binding in 293T cells transfected with the indicated plasmids. (B) Co-immunoprecipitation analyses of c-KIT, Apaf-1 and Hsp90β/phosphor-Hsp90β (pTyr) binding in GIST-T1 cells treated with or without bortezomib. (C) Reciprocal co-immunoprecipitation analyses of mutual interactions of c-KIT, Hsp90β, and Apaf-1 in 293T cells transfected with indicated plasmids and/or incubated with c-KIT purified from GIST-T1 cells.

Discussion

Under physiological conditions, the activation of RTKs is triggered by binding to its ligand SCF, which is necessary to maintain the normal proliferation of cells [40]. Over the past decade, scientists have delineated the central role of RTKs in the pathophysiology of GISTs and that aberrant expression of constitutively activated c-KIT or PDGFR is the main characteristic of GISTs and also the target of TKIs [15]. The implementation of TKIs, including imatinib, sunitinib, and dasatinib, has revolutionized the therapeutic strategy for GISTs. Unfortunately, like the application of TKIs in CML, resistance to TKIs in GISTs occurs quickly [41]. The sensitivity of GISTs to different TKIs is dependent on the specific mutation site on c-KIT or PDGFR [17]. The mutation on c-KIT exon 11 is the most frequent mutation in GISTs and these tumors experience the best response to imatinib therapy; however, the other types of c-KIT mutations often reflect primary resistance to imatinib [16]. Due to acquired secondary mutations that may interfere with drug activity on c-KIT, GISTs eventually become resistant to imatinib. Nevertheless, these observations indicate that secondary resistance of GISTs to RTKs is still dependent on c-KIT-driven and downstream activation. Hence, an alternative strategy to inhibit the c-KIT pathway is still needed. This is in agreement with other potential approaches, including inhibition of c-KIT transcription, genetic alteration of c-KIT, application of monoclonal antibodies, and/or the combination of TKIs and inhibitors of PI3K [28,42,43]. In this study, we showed that BOR, a proteasome inhibitor, significantly enhanced the cytotoxicity of dasatinib in GIST-T1 cells through internalization and subsequent degradation of c-KIT by binding to Cbl. Dasatinib did not induce obvious apoptosis in GIST-T1, but it significantly enhanced the apoptosis induced by BOR.

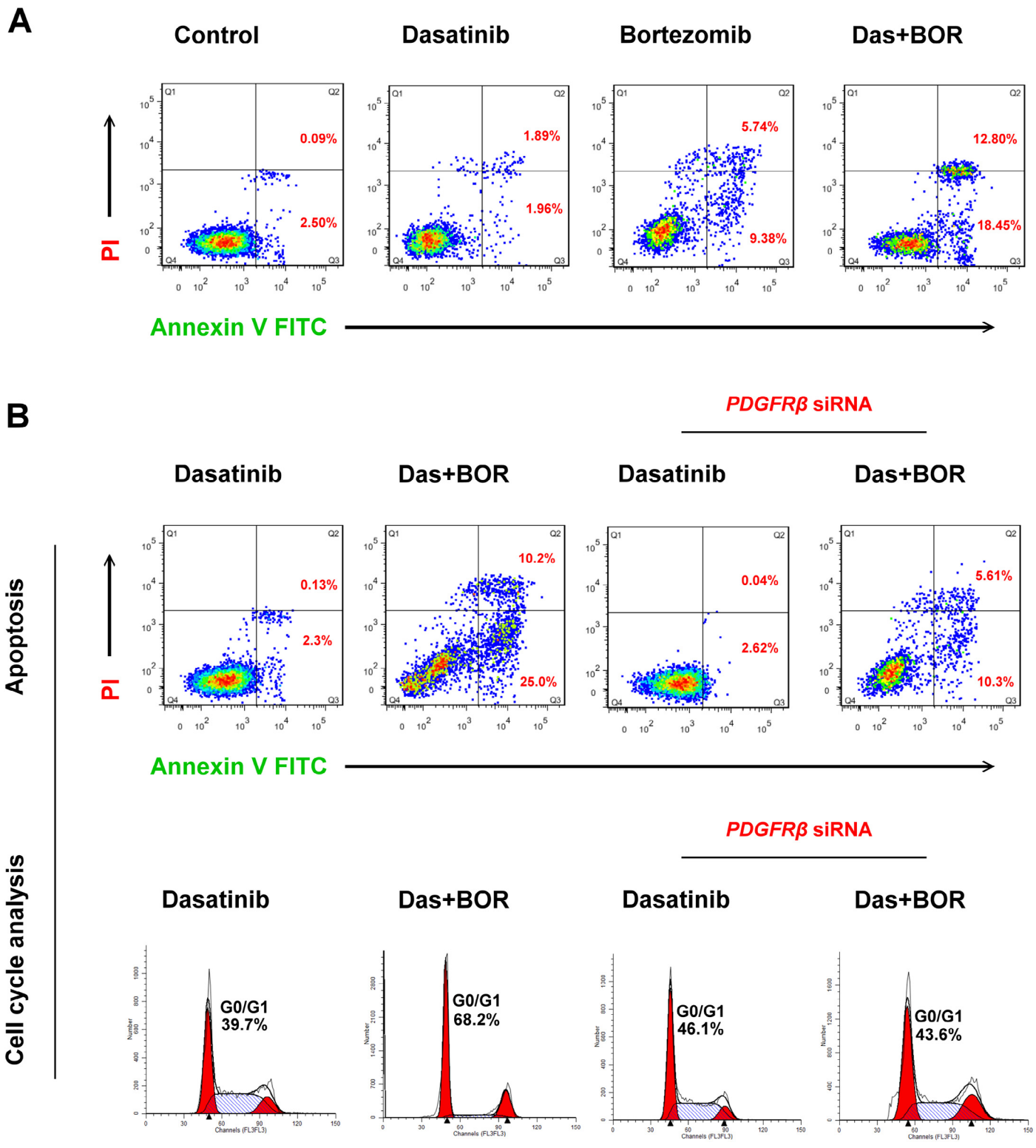

Fig. 5. Dasatinib enhanced the apoptosis induced by bortezomib. (A) Apoptotic analyses of untreated GIST-T1 cells and GIST-T1 cells treated with dasatinib, bortezomib, and dasatinib plus bortezomib (Das + BOR). (B) Apoptotic and cell cycle analyses of GIST-T1 cells and *PDGFRβ* siRNA GIST-T1 cells treated with dasatinib and dasatinib plus bortezomib (Das + BOR).

While binding to SCF, c-KIT will immediately dimerize and internalize in a clathrin-dependent manner [44]. In GIST-T1 cells, we found that BOR-induced internalization of c-KIT was also clathrin-dependent by pretreating cells with DY, which inhibits the GTPase activity of dynamin that disturbs the formation of clathrin-coated transmembrane vesicles. In addition, the retention of c-KIT on cell membranes by DY inhibited BOR-induced apoptosis in GIST-T1 cells, which indicates that internalization of c-KIT is essential for BOR-induced apoptosis. We also found after c-KIT internalization, c-KIT degradation was the downstream gatekeeper for BOR-induced

apoptosis based on the observation that BOR did not induce apoptosis in *Cbl* siRNA GIST-T1 cells. These data suggest that c-KIT might bind to an apoptosis initiator, which can be released when c-KIT is degraded.

Other studies on GIST therapy have shown that an Hsp90 inhibitor decreased the expression of c-KIT and significantly inhibited the growth of GIST cells even harboring the secondary c-KIT mutation [45]. c-KIT might be the substrate client protein as Hsp90 functions as a cellular molecular chaperone. Indeed, in this study, we found that c-KIT could bind and phosphorylate Hsp90β, through which c-KIT indirectly sequestrated Apaf-1. When Apaf-1 was released by BOR treatment, it might initiate the apoptosis cascade by recruiting cytochrome c and dATP [39].

A troublesome question related to the clinical treatment of GISTs is that most individuals undergo a quiescent response to TKIs after initial sensitivity [46]. Therefore, it is important to identify the best approach to kill quiescent cells. In this study, we also found that dasatinib treatment did not induce obvious apoptosis in GIST-T1 cells. However, when treating cells with dasatinib plus BOR, cells underwent dramatic apoptosis, whereas BOR treatment alone only triggered apoptosis in the minority of GIST-T1 cells. Hence, we hypothesize that although dasatinib could not induce apoptosis, dasatinib-induced cell cycle arrest facilitated BOR-induced apoptosis in GIST-T1 cells and the synergetic pro-apoptosis effect was PDGFRβ dependent because dasatinib-induced upregulation of c-KIT was reversed by BOR (Fig. S5), which was further confirmed by PDGFRβ knockdown in GIST-T1 cells. In agreement with other research [47], our data also demonstrated that PDGFRβ functioned as a regulator of cell cycle. In this study, we present a novel therapeutic method to eradicate GIST cells by treating them with a combination of dasatinib and BOR regardless of c-KIT mutation status, which may benefit TKI-resistant GIST patients.

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Conflict of interest

The authors have no conflict of interests.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2015.02.044.

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